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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 12 October 1999 with an application for Letters Patent number 500261 made by JOHN ROBERT TAGG;KAREN PATRICIA DIERKSEN;UNIVERSITY OF OTAGO.

Dated 17 October 2000.

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PATENTS ACT 1953

PROVISIONAL SPECIFICATION

IMPROVEMENTS IN OR RELATING TO LANTIBIOTICS

We, **JOHN ROBERT TAGG, an Australian citizen, of 39 Braeview Crescent, Dunedin, New Zealand; KAREN PATRICIA DIERKSEN, a US citizen, of 129 Tomahawk Road, Dunedin, New Zealand; and UNIVERSITY OF OTAGO, a body corporate first established under the University of Otago Ordinance 1869, of Leith Street, Dunedin, New Zealand,** do hereby declare this invention to be described in the following statement:

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IMPROVEMENTS IN OR RELATING TO LANTIBIOTICS

This invention relates to lantibiotics, microorganisms producing such lantibiotics, and to the uses of both the microorganisms and lantibiotics produced therefrom.

BACKGROUND

Bacterial infection in humans is a problem of both considerable personal concern, and economic importance in the heath field.

Streptococcal infections are particularly prevalent causing ailments ranging from dental caries and minor throat infections to serious diseases such as scarlet fever, rheumatic fever and acute glomerulonephritis.

In order to reduce the incidence of streptococcal infections, it is desirable to control or prevent the growth of the harmful causative bacteria. One approach towards this is to provide lantibiotics active against streptococci and microorganisms capable of producing such lantibiotics, which are suitable for use in controlling or preventing the growth of harmful streptococci bacteria.

Streptococcus salivarius has been found to have a high incidence of lantibiotic production (Dempster RP *et al* (1982) *Arch Oral Biol* 27:151)). One lantibiotic which has been characterised from *S. salivarius* is salivaricin A (Ross *et al* (1993) *Appl Envir Microbiol* 59:2014). However, while demonstrating inhibitory activity against a number of streptococcal species, the activity was bacteriostatic rather than bacteriocidal. Salivaricin A and microorganisms which produce it therefore do not provide a complete answer to controlling streptococcal infections.

The applicants have now identified a further anti bacterial protein. This protein, which the applicants have found to be at least partly bacteriocidal rather than bacteriostatic, is the primary focus of the present invention.

SUMMARY OF THE INVENTION

Accordingly, in one aspect, the present invention may broadly be said to consist in an antibacterial protein which has a molecular mass of approximately 2736 Da as determined by ion-spray mass spectrometry, and the N-terminal amino acid

sequence Gly-Gly-Gly-Val-Ile-Gln, or a functionally equivalent fragment or variant thereof.

The lantibiotic of the invention has been termed by the applicants "salivaricin B".

Conventially, salivaricin B is obtained by expression of a DNA sequence coding therefor in a host cell organism.

In a further aspect, the invention provides a therapeutic formulation comprising salivaricin B as defined above or a functionally equivalent fragment or variant thereof in combination with a diluent, carrier and/or excipient.

In yet a further aspect, the invention provides a therapeutic formulation comprising a microorganism capable of expressing salivaricin B as defined above, or a functionally equivalent fragment or variant thereof, in combination with a diluent, carrier and/or excipient.

Preferably, said microorganism is capable of expressing salivaricin B alone or in combination with a secondary lantibiotic.

Most preferably, said secondary lantibiotic is salivaricin A2.

Conveniently, said microorganism is selected from *S. salivarius* strains K12 and K30.

In a particularly preferred embodiment, the therapeutic formulations are in the form of foodstuffs, most preferably in the form of dairy product-based foodstuffs.

In still a further aspect, the invention provides a microorganism which expresses salivaricin B.

Preferably, said microorganism is selected from *S. salivarius* strains K12 and K30.

In still further aspects of the invention, there are provided methods of treating an individual to at least inhibit growth of harmful streptococcal bacteria in the upper respiratory tract comprising the step of administering an effective amount of salivaricin B orally to said individual.

Preferably, said salivaricin B is administered as part of a therapeutic composition.

Conveniently, in said method said inhibitory effect is caused by colonising at least part of the upper respiratory tract of an individual with a viable microorganism which expresses salivaricin B.

Preferably, said microorganism is administered as part of a foodstuff.

More preferably, said microorganism is a *S. salivarius* strain selected from strains K12 and K30.

In yet a further embodiment, said method includes a preliminary step of pre-treating said individual to at least reduce the bacterial population present in the upper respiratory tract.

Preferably, said pre-treatment comprises the step of administering an antibiotic, preferably erythromycin, orally to said individual.

In yet a further embodiment, the invention provides a method of treatment of a patient against infections of the upper respiratory tract caused by Streptococcal organisms which comprises the steps of:

(i) orally administering to said patient an amount of an antibiotic effective to reduce the numbers of streptococci present; and

(ii) administering, to the resulting bacterially depopulated environment, BLIS producing *S. salivarius* organism(s) to repopulate said environment.

Although the invention is broadly as described above, it will be appreciated by those persons skilled in the art that the invention is not limited thereto but also includes embodiments of which the following description gives examples.

DESCRIPTION OF THE INVENTION

BLIS (bacteriocin-like inhibitory substances) are extracellularly released bacterial peptides or proteins that in low concentrations are able to kill certain other closely related bacteria by a mechanism against which the producer cell exhibits a degree of specific immunity.

The term lantibiotic is a term derived from lanthionine-containing antibiotics (Schnell *et al* Nature 333:276, 1988). Lantiobiotics are a category of BLIS. The lantibiotics are ribosomally synthesised as prelantibiotics, having an N-terminal extension (leader peptide) that is cleaved off by a processing enzyme during formation of the mature (biologically active form) of the molecule. A characteristic feature is that they are polycyclic polypeptides containing lanthionine and/or β-methyl lanthionine, which form thioether bridges within the peptide chain. A classification of the currently reported lantibiotics into two types, A and B, has been proposed by Jung in *Angewandte Chemie* 30:1051-1192, 1991.

Previous investigations by the applicant located a number of BLIS-producing strains of *Streptococcus salivarius* with activity against certain other streptococci. The BLIS produced by one strain, (strain 20P3) was isolated, partially purified and a preliminary characterisation effected. This preliminary characterisation indicated that the BLIS produced was a relatively heat stable protein of molecular mass in the range 3500 to 8000 Da. The BLIS was given the name SAL 20P3.

Subsequent investigations elicited the amino acid sequence of SAL 20P3, together with its molecular mass. The specifically identified lantibiotic was renamed salivaricin A (Ross *et al*, Appl. Envir. Microbiol 59:2014).

The BLIS of the present invention is distinct from salivaricin A. This distinction is both in terms of its molecular mass (2736 Da compared with 2316 Da for salivaricin A) and in terms of its N-terminal amino acid sequence. Salivaricins A and B are also distinct in terms of their inhibitory activity. Specifically, whereas salivaricin A has been found to be effective as a bacteriostat against most strains of *Streptococcus pyogenes*, salivaricin B has been determined to be bacteriocidal. More importantly, no strains of *S. pyogenes* that are resistant to salivaricin B have yet been detected.

Therefore, in a first aspect, the invention is directed to the purified antibacterial protein, salivaricin B. The invention also provides fragments or variants of salivaricin B where they exhibit functional equivalency.

It will be further appreciated that modifications can be made to the native amino acid sequence of both the protein and active fragments thereof while still at least substantially retaining their biological activity. Such modifications to the native amino acid sequence to result in the insertion, substitution or deletion of one or more amino acids are specifically within the scope of this invention.

The protein and fragments of the invention can be prepared in a variety of ways. For example, by isolation from a natural source, by synthesis using any suitable known techniques (such as is described for nisin synthesis by Wakamiya *et al.*, (1991) in "Nisin and Novel Lantibiotics" ed. G. Jung and H. G Shal, 189-203, Escom, Leiden) or as is preferred, through employing recombinant DNA techniques.

The variants of both the native protein and its active fragments can similarly be made by any of those techniques known in the art. For example, variants can be prepared by site-specific mutagenesis of the DNA encoding the native amino acid sequence as described by Adelman *et al.*, DNA 2, 183 (1983).

Where, in the preferred embodiment, recombinant methodology is used to produce the BLIS, it is necessary as a first step to obtain DNA encoding the desired product. Such DNA also comprises an aspect of this invention.

The DNA of the invention may encode the native protein or an active fragment thereof.

The DNA can be isolated from natural sources, for example, from a microorganism in the oral cavity of humans using probes and/or amplification primers based upon the determined amino acid sequence of salivaricin B. The DNA thus identified may be produced as intron free cDNA using conventional techniques. The DNA can also be produced in the form of synthetic oligonucleotides where the size of the active fragments permits, for example by using the phosphotriester method of Matteucci *et al.* J. Am. Chem. Soc. 103:3185-3191, 1981.

Where recombinant methodology is employed, the DNA of the invention may also code for a fusion protein comprising the antibacterial protein or fragment and a vector protein. Generally, this vector protein may be cleaved chemically or enzymatically from the antibacterial protein or fragment according to known techniques.

The invention also contemplates variants of the protein and its fragments which differ from the native amino acid sequences by the insertion, substitution or deletion of one or more amino acids. Where such a variant is desired, the nucleotide sequence of the native DNA is altered appropriately. This alteration may be made

through elective synthesis of the DNA or by modification of the native DNA by, for example, site-specific or cassette mutagenesis.

Preferably, where portions of cDNA or genomic DNA require sequence modifications, site-specific primer directed mutagenesis is employed. This technique is now standard in the art.

Once obtained, the modified DNA is treated to be suitable for insertion into the appropriate cloning and/or expression vector. To this end the DNA is cleaved, tailored and religated as required.

Cleavage is performed by treatment with restriction enzymes in a suitable buffer. Any of the large number of commercially available restriction enzymes can be used in the manner specified by the manufacturer. After cleavage, the nucleic acid is recovered by, for example, precipitation with ethanol.

Tailoring of the cleaved DNA is performed using conventional techniques. For example, if blunt ends are required, the DNA may be treated with DNA polymerase 1 (Klenow), phenol and chloroform extracted, and precipitated by ethanol.

Relegation can be performed by providing approximately equimolar amounts of the desired components, appropriately tailored for correct matching, and treatment with an appropriate ligase (e.g. T_4 DNA ligase).

The DNA molecule thus obtained is inserted into a cloning vector at a location which permits the protein product for which it codes to be expressed.

Suitable cloning vectors may be constructed according to well known techniques, or may be selected from the large number of cloning vectors available in the art. While this cloning vector selected may vary according to the host cell intended to be used for expressing the BLIS-encoding DNA, useful cloning vectors will generally have the following characteristics:

(i) the ability to self-replicate;

(ii) possession of a single target for any particular restriction endonuclease; and

(iii) desirably, carry genes for a readily selectable marker such as antibiotic resistance.

Two major types of cloning vectors possessing the aforementioned characteristics are plasmids and bacterial viruses (bacteriophages or phages). Examples of suitable cloning vectors include pUC18, Mp18, Mp19, pRB322, pMB9, ColE1, and pCR1 from *E. coli*; wide host range plamids including RP4, phage DNA's, such as lambda and M13 and shuttle vectors such as pSA3 and pAT28.

For expression of the BLIS-encoding DNA in the host, the cloning vector must also incorporate an expression control sequence. A typical expression control sequence can be described in terms of five elements. In the order in which they appear in the gene, the elements are as follows:

(a) the promoter region;
(b) the 5' untranslated region (signal or leader sequence);
(c) the protein coding sequence;
(d) the 3' untranslated region; and
(e) the transcription termination region.

The function of each of these elements is well recognised.

Any of a wide range of such control sequences can be used including, for example, those from the lipoprotein gene, the β-galactosidase gene, the tryptophan gene, the β-lactamase gene, and phage lambda.

As element (c), the DNA sequence coding for the lantibiotic is inserted into the cloning vector control sequence in the manner indicated above.

An appropriate host into which the cloning vector is to be inserted is then selected. Potentially useful hosts include bacteria, yeasts, fungi, insect, animal and plant cells. Procaryotic hosts are generally preferred for the present invention. Non-disease causing bacterial hosts are particularly suitable.

Bacterial hosts are generally selected from among the gram positive bacteria. *Streptococcus* hosts are preferred for use in the present invention.

As will be appreciated, in the selected host system, plasmid vectors that contain replication sites and control sequences derived from a species compatible with the host are used.

The cloning vector formed as above is used to transform the selected host, again using techniques well known in the art, for example, the calcium chloride treatment as described by Cohen, S.N. Proc. Nat. Acad. Sci. 69, 2110, 1972.

Upon transformation of the selected host with the cloning vector, the protein or fragment encoded can be produced, potentially as a fusion protein, by culturing the host cells. The exogenous protein product or fragment is then isolated using routine methods such as freeze-thaw extraction. Purification is effected as necessary using conventional techniques.

The purified protein is then available for use therapeutically.

Therefore, in a further aspect, the present invention is directed to therapeutic formulations suitable for use in the treatment or prevention of microbial infections, particularly streptococcal infections. The formulations are particularly suitable for use against *S. pyogenes* and *S. sobrinus*. These therapeutic formulations comprise salivaricin B or a fragment or variant thereof in combination with a diluent, carrier or excipient therefor, such as are known in the art. Examples of therapeutic formulations in which salivaricin B can be employed include lozenges, syrups, mouth washes, gargles, toothpastes, and mouth sprays but are not limited thereto.

In a further aspect the present invention provides a therapeutic formulation comprising a non-disease-causing viable microorganism capable of colonising the upper respiratory tract or a part thereof and expressing the lantibiotic of the invention, in combination with a carrier, diluent and/or excipient.

In one embodiment the microorganism is a transformed host microorganism produced in accordance with the invention. It is, however, preferred that the microorganism be one which produces salivaricin B as a native product. Examples of such microorganisms are *S. salivarius* strains MIN5, P, K12, and K30, with strains K12 and K30 being particularly preferred.

S. salivarius strains K12 and K30 have been deposited with Deutsche Sammlung von Mikroorganismen Und Zellkulturen GmbH, Mascheroder Weg 1 b, D-38124 Braunschweig, Germany.

S. salivarius strains K12 and K30 have been determined as expressing not only salivaricin B but also salivaricin A2. Salivaricin A2 is related but not identical to salivaricin A. The full sequences for salivaricin A and salivaricin A2 are as follows:

	-9	-8	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8	9	10
sal A	Lys	Glu	Leu	Met	Glu	Val	Ala	Gly	Gly	Lys	Arg	Gly	Ser	Gly	Trp	Lle	Ala	Thr	Lle
sal A 2	Lys	Glu	Leu	Met	Glu	Val	Ala	Gly	Gly	Lys	Arg	Gly	Thr	Gly	Trp	Phe	Ala	Thr	Lle

	11	12	13	14	15	16	17	18	19	20	21	22	23
sal A	Thr	Asp	Asp	Cys	Pro	Asn	Ser	Val	Phe	Val	Cys	Cys	stop
sal A 2	Thr	Asp	Asp	Cys	Pro	Asn	Ser	Val	Phe	Val	Cys	Cys	stop

In this embodiment, it is preferred that the therapeutic formulations of the invention are in the form of a foodstuff or drink. It is particularly preferred that the foodstuff or drink be a dairy product-based foodstuff or drink, including by way of example, yoghurt, cheese, milk and flavoured milks.

A particularly preferred formulation is where freeze-dried strains of salivaricin B-producing *S. salivarius* be included in milk powder formulations in a manner similar to that previously reported for the preparation of Bifidus Milk Powder (Nagawa *et al* (1988); J Dairy Sci 71:1777-1782).

Various aspects of the invention will now be illustrated in a non-limiting way by reference to the following experimental section.

Experimental

Extraction of salivaricin

Salivaricin B was purified from lawn cultures of the test strains *S. salivarius* K12 and K30 grown for 18 hours at 37°C in a 5% carbon dioxide in air atmosphere on M17 medium supplemented with 0.5% Davis agar, 0.5% sucrose, 0.5% human plasma and 0.1% calcium carbonate. The lawn cultures were inoculated by swabbing on to the surface of the agar medium from an 18 hour 37°C Todd Hewitt broth culture of the producer strain. Extraction of the salivaricin B activity is achieved by freezing

the agar plates at -70°C and then thawing at room temperature to collapse the agar gel, followed by centrifugation to clarify the extracted liquor. The titre of inhibitory activity in these freeze/thaw extracts is generally 2-4 AU/ml.

Titration of salivaricin activity

Salivaricin activity is titrated using an agar surface assay. Drops (20ul) of two-fold saline dilutions of the sample are assayed against *Microoccus luteus* T-18 on Columbia agar base. The reciprocal of the highest dilution to produce a definite zone of inhibition of the growth of the indicator lawn is the titre in arbitrary units per ml (Au/ml) of salivaricin activity.

Purification of salivaricin B

A two-litre volume of freeze thaw extract was applied to an XAD-2 column (diameter 5.0cm, bed volume 150 ml; Serva) and washed with 7 bed volumes of 50% (v/v) methanol. Salivaricin activity was eluted with 5 bed volumes of 90% (v/v) methanol (adjusted to pH2 with 11.6 M CH1) and concentrated by evaporation at 50°C under reduced pressure. Aliquots (1-ml) of this material were applied to a Brownlee C8 reverse phase column (Aquapore RP 300; pore size, 7um; 30 by 4.6mm; Applied Biosystems, Inc.), equilibrated with 0.1% trifluoroacetic acid (TFA). Fractionation of this material is achieved by using a Pharmacia fast protein liquid chromatography (FPLC) system at a flow rate of one ml per minute using a 10 minute gradient (0 to 28% acetonitrile containing 0.085% TFA) followed by 80 minute isocratic (28% acetonitrile) elution. During this isocratic elution, phase separation is achieved of salivaricin A (elution starting at around 40 minutes) and salivaricin B (starting at around 60 minutes). Each 1-ml fraction was tested for inhibitory activity against M.*luteus* T18. The active fractions in each region corresponding to salivaricin A and salivaricin B were separately pooled. Each pool was lyophilized and then dissolved in 0.1% TFA. Aliquots of each of these preparations were then loaded onto a C_{18} reversed-phase High Pressure Liquid Chromatography (HPLC) column (Alltech Nucleosil C_{18}; 10 um; 250.0 x 4.6 mm) equilibrated with 0.1% TFA and further fractionated using a Waters/Millipore HPLC system by application of appropriate gradients of acetonitrile:

Salivaricin A was eluted as a homogeneous peak with 34-35% acetonitrile and salivaricin B with 38-40% acetonitrile. Absorbence was monitored at 214 mm and fractions corresponding to the various peaks were collected manually. Inhibitory

activity was detected by a spot diffusion teat using *M. luteus* T-18 as the indicator. The active fractions from each run were pooled, lyophilised and redissolved in 1 ml of Milli Q™-purified water containing 0.1% TFA. The fractions containing inhibitory activity (purified salivaricin) were pooled and stored at -20°C.

Ion-spray mass spectrometry indicated that the molecular mass of salivaricin B was 2736 Da. Edman analysis of purified salivaricin B revealed the N-terminal sequence Gly-Gly-Gly-Val-Ile-Gln.

Antibacterial activity of salivaricin B

Strains of *S. salivarius* such as 20P3 and 5 that produce salivaricin A (but not salivaricin B) inhibit all of 9 standard indicator bacteria other than indicator 3.. This pattern of inhibition in code form is known as production (P) type 677. By contrast, strains K12 and K30 that produce both salivaricin A2 and salivaricin B inhibit the growth of all 9 standard indicators, activity referred to as P-type 777. The P-typing test involves first growing the test strain on blood agar as a diametric streak culture. After removing this growth, the agar surface is sterilizied with chloroform vapour, aired and the 9 standard indicator bacteria (including 4 strains of *Streptococcus pyogenes*) are cross-streaked across the line of the original test strain inoculum. Following incubation, interference with growth of the indicators in the vicinity of the original producer streak is taken as indicative of bacteriocin activity. In the case of strains 20P3 and 5 (producers of salivaricin A) the inhibitory activity can be shown to be bacteriostatic ie viable indicator cells can be recovered in large numbers from the inhibition zone by sampling with a swab and transferring the cells to a fresh (non-bacteriocin-containing) agar medium. By contrast, the effect of the P-type 777 strains (shown also to produce salivaricin B) is bactericidal against the standard indicators ie no viable cells can be recovered from the inhibition zone in deferred antagonism tests. Furthermore, tests using purified preparations of salivaricin A and salivaricin B (data not shown) have confirmed that the action against *S. pyogenes* of salivaricin A is bacteriostatic whereas that of salivaricin B is bactericidal.

Discussion

The results above demonstrate the inhibitory and bacteriocidal effect of salivaricin B and microorganisms which produce this BLIS. Salivaricin B and/or microorganisms which produce it are therefore applicable in methods of treating individuals against

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the harmful effects of streptococcal infections in the upper respiratory tract, including the mouth. These methods include methods of treatment of conditions such as streptococcal sore throats (caused mainly by *pyogenes*) and dental caries (caused by *S. sobrinus*).

The presently preferred administrable formulations are blends of freeze-dried *S. salivarius* strains with skim milk powder or the like which has been flavoured to enhance palatability.

Indications to date are that such formulations are effective when reconstituted by addition of water and sipped on three to four occasions during the course of the day, such that a total of 50 mls of the flavoured product is consumed (containing approximately 2 x 10^7 cells/ml of freeze-dried *S. salivarius* organism(s)).

Where the freeze-dried *S. salivarius* strains are selected from K12 and K30, there is the added advantage that salivaricin B is expressed together with salivaricin A2. Co-expression of these two BLIS renders the formulation particularly bacteriocidal in relation to *S. pyogenes* and *S. sobrinus*.

It will be appreciated that the above description is provided by way of example only and that variations in both the materials and the techniques used which are known to those persons skilled in the art are contemplated.

WEST-WALKER BENNETT

per M+Hampton

ATTORNEYS FOR THE APPLICANT

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